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# HIGH DOSES OF COBALT INHIBITED HAIR FOLLICLE DEVELOPMENT IN REX RABBITS

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**Abstract:** An experiment was conducted to investigate the effect of cobalt supplementation on hair follicle development in rabbits. Rex rabbits (30-d-old, n=180) were divided randomly into five equal treatment groups: rabbits fed a basal diet (control, measured cobalt content of 0.27 mg/kg) or rabbits fed a basal diet with an additional 0.1, 0.4, 1.6 or 6.4 mg/kg cobalt (in the form of cobalt sulfate) supplementation (measured cobalt contents of 0.35, 0.60, 1.83 and 6.62 mg/kg, respectively). Treatment with 6.4 mg/kg cobalt significantly decreased hair follicle density ($P<0.05$), while low levels of cobalt (0.1-1.6 mg/kg) had no effect on hair follicle density ($P>0.05$). The addition of dietary cobalt at the highest level examined (6.4 mg/kg) significantly increased the gene expression of bone morphogenetic protein (BMP) 2 and BMP4 in skin tissue ($P<0.05$), while the mRNA levels of versican, alkaline phosphatase, hepatocyte growth factor, and noggin remained unchanged ($P>0.05$). Compared with their levels in the control group, dietary cobalt treatment significantly suppressed the protein levels of p-mechanistic target of rapamycin (mTOR) and p-ribosomal protein S6 protein kinase ($P<0.05$) but did not alter the protein levels of p-AMP-activated protein kinase, Wnt10b or p-β-catenin ($P>0.05$). In conclusion, cobalt at the highest concentration examined inhibited hair follicle development, which may have involved the mTOR-BMP signalling pathway.

**Key Words:** cobalt, hair follicle development, mTOR-BMP signalling pathway, rabbits.

## INTRODUCTION

The Rex rabbit is an important small herbivore for fur production. The quality of Rex rabbit fur is largely dependent on rabbit hair density. Paus and Cotsarelis (1999) indicated that hair follicle density can determine hair density. Hair follicles are a complicated tissue consisting of multiple layers of various types of cells (Roges 2004). The development of hair follicles can be divided into three phases (anagen, the growth phase; catagen, the cessation phase; and telogen, the rest phase) (Stenn and Paus 2001). Hair follicles exist in large quantities and have unique repetitive regenerative behaviours (Marlon *et al.,* 2009). At telogen, hair follicle cells may resume anagen development after stimulation by certain factors. These cycles continue repetitively throughout the entire lifetime of rabbits. The induction of hair follicles, maintenance of hair shaft growth and differentiation of undifferentiated cells in the shaft are controlled by specific genes, such as bone morphogenetic protein (BMP), versican, alkaline phosphatase (ALP), hepatocyte growth factor (HGF), and noggin (Madaan *et al.,* 2018). Versican, ALP, HGF, noggin and tyrosinase positively regulate hair follicle growth, but BMP2 and BMP4 have negative effects on hair follicle growth. Hair follicle growth can also be affected by certain signalling pathways. The AMP-activated protein kinase (AMPK) family, an important energy sensor, regulates the activity of many key enzymes related to the hair development cycle (Poeggeler *et al.,* 2010). Wnt signalling molecules in the three stages of hair follicle growth

were detected by polymerase chain reaction (PCR) microarray (Li *et al.,* 2011). Wnt10b may promote hair follicle growth by inducing the switch from telogen to anagen (Li *et al.,* 2011). Mechanistic target of rapamycin (mTOR) signalling could regulate stem cell activation during hair regeneration.

Hair follicle development is sensitive to mineral elements in the diet, and previous studies have demonstrated the role of copper and zinc in the production of wool and hair from follicles (Li *et al.,* 2011). However, the effect of another mineral, cobalt, on hair follicle growth is still unknown. John and Gordon (1947) found that rabbits fed whole milk and corn grain required less than 0.1 µg of cobalt per animal per day, but cobalt is essential for rabbit growth. It is well known that cobalt is involved in animal physiology due to its essential role as a component of the cobalamin molecule (vitamin B12), which reportedly influences hair follicle growth and cycling in humans, but the mechanisms of this effect are unclear (Krugluger *et al.,* 2011). Vitamin B12 deficiency is accompanied by hyperpigmentation of the skin and hair (Wolff, *et al.,* 1998). Vitamin B12 is not added to a rabbit's diet, and vitamin B12 levels in basic ingredients could meet normal requirements. Soil in the Sichuan Province of China, which has the largest number of rabbits, is rich in cobalt, which produces a high level of cobalt in pastures. The rabbit diet compounded with a high level of cobalt in pastures in the Sichuan Province may be a toxic risk factor. In the present study, we investigated the effect of high levels of dietary cobalt on hair follicle growth and the related signalling pathways and considered the possible pathways related to the effect of cobalt on hair follicle growth. Finally, our results reveal that high levels of dietary cobalt are a toxic risk factor for hair follicle development which serve as an important guide for the feeding of Rex rabbits.

## MATERIALS AND METHODS

### *Animals*

Rex rabbits (30-day-old) were individually housed in plastic cages (size of cage: 60×40×40 cm) in a closed building (maximum temperature, 25°C; minimum temperature, 20°C; 12/12 light/dark cycle). Rabbit diets were formulated according to standard values for rearing rabbits set forth by de Blas and Mateos (1998) and pressure-pelleted (4 mm diameter). The diet ingredients and chemical composition are listed in Table 1. All rabbits had free access to feed and water during the rearing period. This study was approved by the Shandong Agricultural University and conducted in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China).

Table 1: Basal diet composition and nutrient levels (% in fresh basis).

| Ingredients | | Nutrient levels[2] | |
|---|---|---|---|
| Corn | 7.00 | Digestible energy (MJ/kg) | 10.11 |
| Wheat middling | 16.00 | Crude protein | 17.37 |
| Corn germ meal | 15.00 | Ash | 19.54 |
| Sunflower meal | 12.50 | Ether extract | 3.14 |
| Rice hulls | 13.00 | Crude fibre | 19.54 |
| Guinea grass | 20.00 | Neutral detergent fibre | 41.59 |
| Soybean meal | 8.00 | Acid detergent fibre | 22.58 |
| Extruded soybean meal | 4.00 | Acid detergent lignin | 7.57 |
| Soybean oil | 0.75 | Calcium | 1.00 |
| Soybean phosphatides | 0.75 | Phosphorus | 0.54 |
| Premix[1] | 3.00 | Lysine | 0.56 |
| Total | 100.00 | Methionine | 0.23 |
| | | Cobalt (mg/kg) | 0.27 |

[1]Premix provided the following per kg of diet: vitamin A 12 000 IU, vitamin D3 900 IU, vitamin E 50 mg, vitamin K3 1.5 mg, vitamin B1 1.5 mg, vitamin B2 5 mg, vitamin B3 40 mg, vitamin B5 50 mg, vitamin B6 0.5 mg, vitamin B11 2.5 mg, choline 600 mg, biotin 0.2 mg, potassium 7 mg, magnesium 3 mg, iron 60 mg, zinc 60 mg, copper 40 mg, manganese 9 mg, iodine 1 mg, stone powder 15 000 mg, sodium chloride 5000 mg, lysine 1000 mg, methionine 2000 mg.
[2]Digestible energy was calculated according to the nutrition levels of ingredients by de Blas and Mateos (1998), while the other nutrient values were measured.

### *Experimental protocol and sample collection*

One hundred and eighty 30-day-old rabbits of a similar body weight (1050±30 g) were divided randomly into five groups as follows (36 replicates per group, half males and half females, and 1 rabbit per replicate): rabbits fed a basal diet (control, measured cobalt content of 0.27 mg/kg) or rabbits fed a basal diet with the addition of 0.1, 0.4, 1.6 or 6.4 mg/kg cobalt (measured cobalt contents of 0.35, 0.60, 1.83 and 6.62 mg/kg, respectively) in the form of feed-grade cobalt sulfate. The experiment lasted for 8 wk, which included a 1-wk adaptation period and a 7-wk experimental period. Feed intake was recorded daily, and body mass was recorded weekly. At the end of the trial, 32 rabbits (8 rabbits per group, half males and half females) were electrically stunned (70 V, pulsed direct current, 50 Hz for 5 s) and sacrificed by cervical dislocation. Mediodorsal skin samples were collected from each rabbit and stored at 4°C for subsequent haematoxylin-eosin (HE) staining of hair follicle slices. In addition, hair follicle cells were separated from another mediodorsal skin sample according to a previously published method (Weinberg *et al.,* 1993), frozen in liquid nitrogen and stored at –70°C for 2 wk for subsequent RNA and protein analyses.

### *Measurements*

Feed intake over the whole trial period was recorded daily and body mass was recorded weekly. Average feed intake and body weight gain were the average daily feed intake and body weight gain, respectively. At the end of the trial, the lengths of ten hairs from skin on the shoulders, medium dorsal area and haunch of each rabbit were measured from the skin to the hair tip. The mean length of ten hairs at every site was calculated as the hair length, and the mean lengths of hairs at the three sites were calculated as the average rabbit hair length. The dietary cobalt content was determined by atomic absorption spectrophotometry according to the methods of Ghaedi *et al.,* (2007).

Skin specimens were observed by histological staining as described in Wang *et al.* (2000). Briefly, skin specimens were fixed conventionally in 4% formaldehyde, dehydrated and embedded in paraffin. Sections with a thickness of 4 μm were stained with haematoxylin and eosin (Sigma-Aldrich, St. Louis, MO). The sections were then examined under an Olympus CX-41 phase contrast microscope (Olympus, Tokyo, Japan).

Total RNA extraction and quantitative real-time PCR were performed as described previously (Liu *et al.,* 2017; Liu *et al.*, 2019). Sequences of the primers used are shown in Table 2. Real-time PCR was conducted using an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster, CA, USA). The PCR data were analysed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The mRNA levels of target genes were normalised to glyceraldehyde

**Table 2:** Gene-specific primers used for the analysis of rabbit gene expression.

| Gene | Genebank accession No. | Primer sequence (5'-3') | Product size/bp |
|------|------------------------|-------------------------|-----------------|
| GAPDH | NM_001082253 | F: TGCCACCCACTCCTCTACCTTCG<br>R:CCGGTGGTTTGAGGGCTCTTACT | 163 |
| β-actin | NM_001101683.1 | F: CGCAGAAACGAGACGAGATT<br>R: GCAGAACTTTGGGGACTTTG | 169 |
| Noggin | XM_002719279 | F: CCAGCACTACCTCCACATCC<br>R: GCGTCTCGTTCAGATCCTTC | 123 |
| ALP | XM_017346489.1 | F: TGCACAGAGCAAGAGAAGGA<br>R: TCTCCCAGGAACAGGATGAC | 125 |
| Versican | XM_017344567 | F: AGGTCAGCCCTCTCAAGACA<br>R: TCTGTTCTTCCCGAGTGGTC | 119 |
| BMP4 | NM_001195723 | F: ACCTCAACTCCACCAACCAC<br>R: CATCCAGGTACAGCATGGAG | 120 |
| HGF | NM_001168707 | F: TTGTCCTCTTGCTCGTTGTG<br>R: GTTCGTGTTGGAATCCCATT | 120 |
| BMP2 | NM_001082650 | F: GGTTTGTGGTGGAAGTGACC<br>R: AGTTACGAGCAAAGGCCTGA | 125 |

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; HGF, hepatocyte growth factor.

3-phosphate dehydrogenase (GAPDH) and β-actin mRNA levels (ΔCT). Based on the CT values, GAPDH and β-actin mRNA levels were stable across the treatments administered in this study ($P>0.1$).

The protein concentration was determined using a BCA assay kit (Beyotime, Jiangsu, China). After boiling at 100°C, protein extracts (80 μg) were electrophoresed in 7.5-10% sodium dodecyl sulfate polyacrylamide gels following the procedure described by Fu *et al.* (2018). The separated proteins were then transferred onto nitrocellulose membranes at 100 V at 4°C. Then, the membranes were stripped to assay phosphorylated proteins and actin separately. The membranes were blocked and immunoblotted with the following primary antibodies (Cell Signaling Technology Inc., Beverly, MA, USA): p-AMPKα[Thr172] antibody, AMPKα antibody, Wnt10b antibody, β-catenin antibody, p-β-catenin[Ser675] antibody, mTOR antibody, p-mTOR[Ser2448] antibody, ribosomal protein S6 protein kinase (p70S6K) antibody, and p-p70S6K[Thr389] antibody. Protein detection following incubation with HRP-labelled goat anti-mouse IgG (H+L) secondary antibody was performed by enhanced chemiluminescence using western blotting detection reagents (Beyotime). A monoclonal mouse anti-tubulin antibody was used as a loading control. Western blots were developed and quantified with BioSpectrum 810 with VisionWorksLS 7.1 software (UVP LLC, California, USA).

### Statistical analysis

The data are presented as the means±standard error of the means. All the data collected were subjected to one-way ANOVA with the Statistical Analysis Systems software package (Version 8e, SAS Institute, Cary, NC) and the primary effect of cobalt treatment was evaluated. The homogeneity of variances among groups was confirmed using Bartlett's test (SAS Institute). The significance of differences in body weight gain, feed intake, hair length and hair follicle density following different treatments was determined using the Student-Newman-Keuls test. For PCR and western blotting data analysis, the significance of differences following different treatments was determined using the paired t-test. Significance was assumed at $P<0.05$.

## RESULTS

The dietary cobalt content did not significantly affect average body weight gain or hair length (Figures 1A and C, $P>0.05$). Although the addition of 0.4 mg cobalt/kg to the diet increased feed intake, the dietary addition of 1.6-6.4 mg cobalt/kg decreased feed intake (Figure 1B, $P<0.05$). The dietary addition of cobalt (6.4 mg/kg) significantly decreased hair follicle density (Figure 1B, $P<0.05$), but lower levels of cobalt (0.1-1.6 mg/kg) did not affect hair follicle density ($P<0.05$).

By studying the expression of genes and proteins related to hair follicle development, we found that the dietary addition of the highest level of cobalt (6.4 mg/kg) significantly increased expression of the BMP2 and BMP4 genes in hair follicle cells (Figures 2E-F, $P<0.05$) but did not change the mRNA levels of versican, ALP, HGF, or noggin (Figures 2A-D, $P>0.05$). Dietary cobalt treatment (6.4 mg/kg) significantly decreased the protein levels of p-mTOR and p-p70S6k (Figures 3D-E, $P<0.05$) but did not alter the protein levels of p-AMPK, Wnt10b or p-β-catenin (Figures 1A-C, $P>0.05$).

## DISCUSSION

We investigated the effect of cobalt on the production performance and development of hair follicles. Although cobalt addition did not significantly affect body weight gain in rabbits, it significantly affected feed intake, which is consistent with the results of a previous study (Schwarz *et al.,* 2000). Cobalt is a trace element essential for the production of vitamin B12 by intestinal microbes to meet vitamin B12 requirements in rabbits (McDowell, 1992). Low levels of cobalt (0.4 mg/kg) increased feed intake compared to that in the control group, indicating a B12 deficiency in the basic rabbit basic. A high level of cobalt (1.6-6.4 mg/kg) decreased feed intake, indicating that the additional dose of cobalt exceeded the normal levels of cobalt required of rabbits. Low levels of cobalt had no significant effect on hair follicle density, indicating that cobalt, which acts as a cofactor for vitamin B12 when present at low levels in the rabbit diet, is useless for hair follicle development. This result is not in agreement with the results of earlier studies showing the effects of copper and zinc. Hynd (2000) found that low levels of supplemented copper or zinc could improve hair follicle development. Moreover, we found that a high cobalt level (6.4 mg/kg) significantly decreased the density of

hair follicles and feed intake, indicating that cobalt is toxic when there is more cobalt than cobalt-binding sites in vitamin B12. To study the inhibitory effect of cobalt on hair follicle development, we measured the levels of related genes and proteins in skin samples from the high cobalt treatment and control groups.

The growth and differentiation of postnatal hair follicles are controlled by reciprocal interactions between the dermal papilla and surrounding epidermal hair precursors. BMP signalling has been implicated in the regulation of both
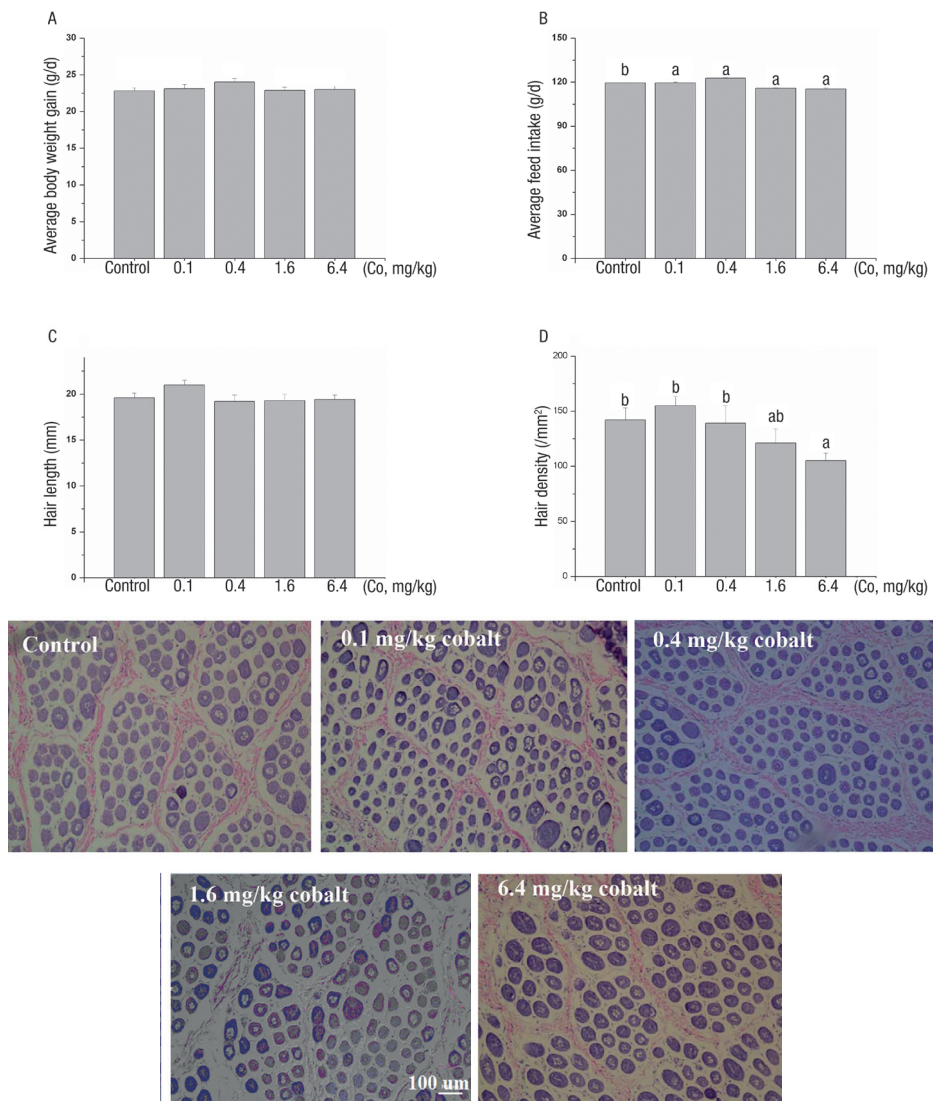


Figure 1: Effects of dietary cobalt on average gain in body weight (A), average feed intake (B), hair length (C) and hair follicle density (D) (E, haematoxylin-eosin staining of hair follicle slices). Values are shown as the mean±standard error of the mean (for gain in body weight and feed intake, n=36; for hair length and fair follicle density, n=8). [a, b, c] Values with different superscripts are significantly different ($P<0.05$).
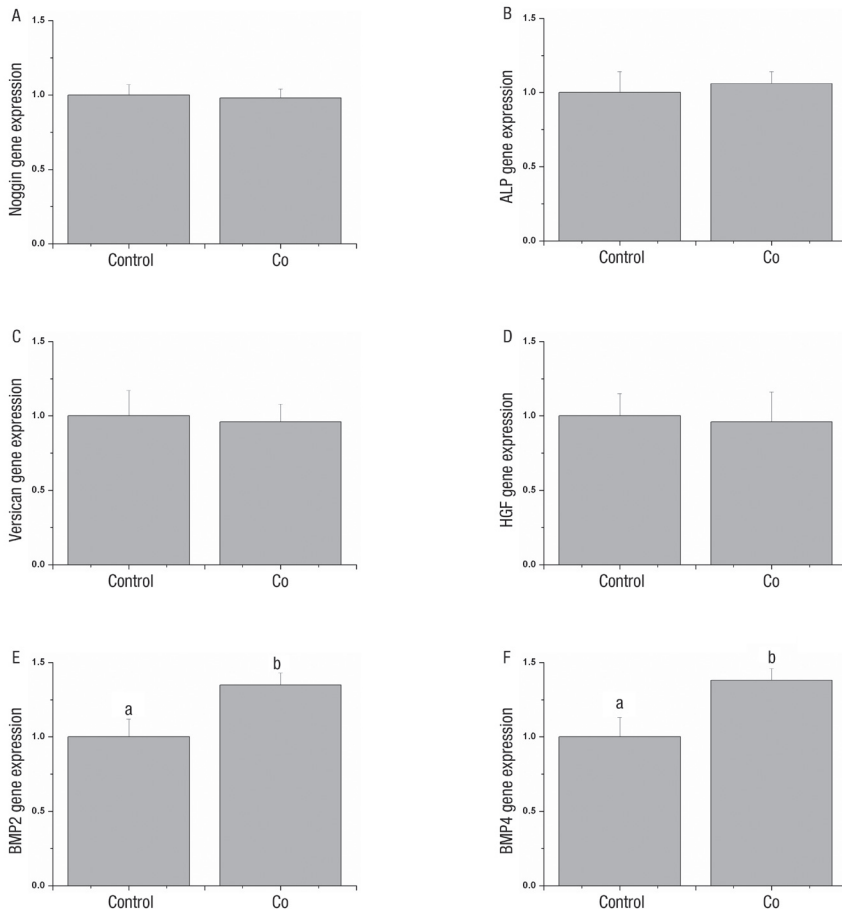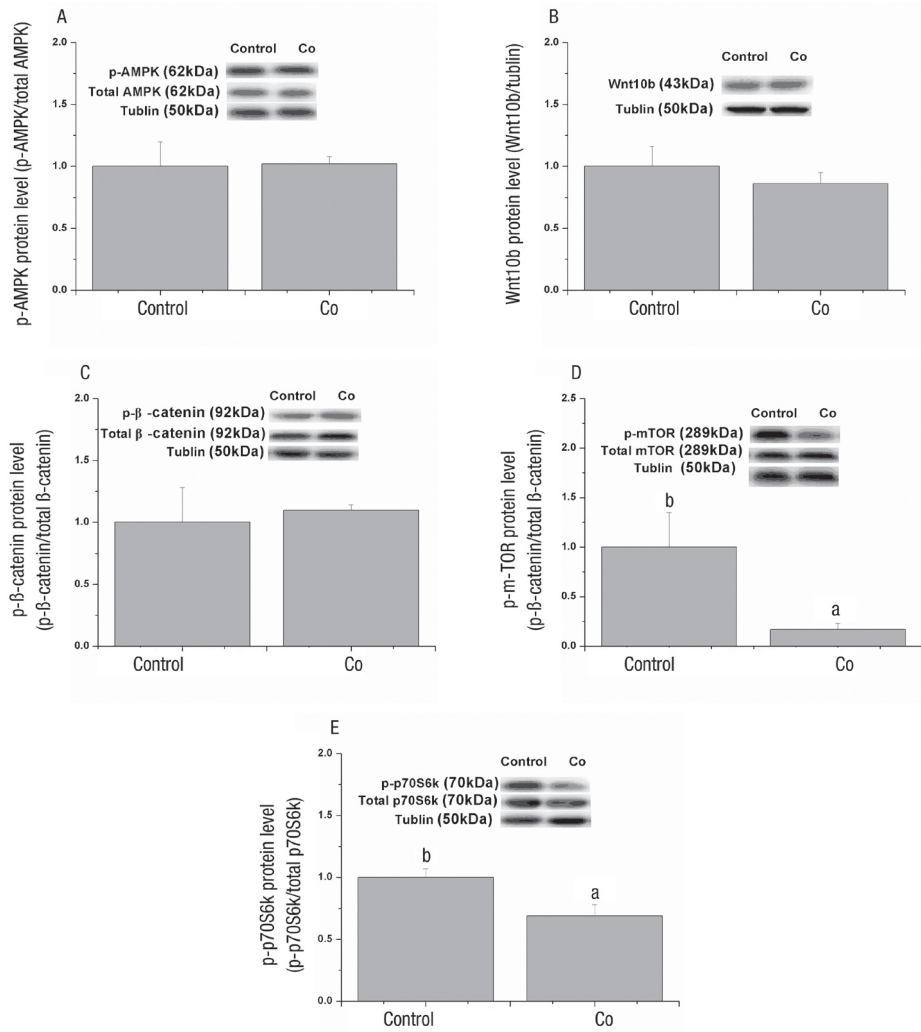
**Figure 2:** Effects of high levels of cobalt on the expression of genes related to hair follicle development. Values are shown as the mean±standard error of the mean (n=8).

[a, b] Values with different superscripts are significantly different ($P<0.05$). ALP, alkaline phosphatase; BMP, bone morphogenetic protein; HGF, hepatocyte growth factor.

proliferation and differentiation in the hair follicle. Several BMP family members are expressed in the postnatal hair follicle (Wilson *et al.,* 1999). BMP had a strong negative effect on hair follicle development. BMP4 transgenic mice showed retarded hair follicle development (Blessing *et al.,* 1993) and the administration of BMP inhibitors improved hair follicle development (Rendl *et al.,* 2008). Treatment with high levels of cobalt significantly repressed the gene expression of BMP4 and BMP2, indicating that BMP2/4 may be important gene targets in the inhibition of hair follicle development by high levels of cobalt. Additionally, the expression of genes that positively regulate hair follicle development (e.g., versican, ALP, HGF and noggin) was not altered after treatment with high levels of cobalt, indicating that versican, ALP, HGF and noggin genes may not be major targets in the regulatory effect of high levels of cobalt on hair follicle development.

AMPK, a serine/threonine protein kinase, is a pivotal regulator that balances cellular energy metabolism and regulates pathological processes and serious diseases. Recent studies have proposed AMPK as a new treatment for alopecia.

**Figure 3:** Effects of high levels of cobalt on the expression of proteins related to hair follicle development. Values are shown as the mean±standard error of the mean (n=8).

[a, b] Values with different superscripts are significantly different ($P<0.05$). AMPK, AMP-activated protein kinase; mTOR, mechanistic target of rapamycin; p70S6K; ribosomal protein S6 protein kinase.

Metformin, an AMPK activator, promotes hair growth *in vitro* by affecting human dermal papilla cells and outer root sheath cells (Chong, 2016). However, we found that the level of p-AMPK protein did not respond to treatment with high levels of cobalt, indicating that AMPK signalling may not be the major signalling pathway by which high levels of cobalt repress hair follicle development. This result is inconsistent with those of previous studies, which showed that cobalt administration enhanced AMPK activation in a dose-dependent manner in the liver, skeletal muscle and white adipose tissue (Kawakami *et al.,* 2012) and p-AMPK protein expression in H9c2 cardiomyoblasts (Gallo *et al.,* 2014). All these results suggest that cobalt affects p-AMPK protein levels in a tissue-specific manner.

Recent data have confirmed the results of previous studies suggesting a fundamental role for the Wnt/β-catenin signalling pathway in hair development (Fuchs *et al.,* 2001). Wnt10b is expressed in placodes at the onset of hair follicle morphogenesis (Reddy *et al.,* 2001). Conditional disruption of β-catenin in the epidermis led to the failure to induce both primary and secondary hair follicles (Huelsken *et al.,* 2001). Cobalt-mimicked hypoxia was shown to promote osteoblast proliferation by increasing β-catenin and Wnt gene expression (Li *et al.,* 2016). In our study, treatment with high levels of cobalt did not significantly alter Wnt10b and β-catenin protein expression, suggesting that the wnt10b/β-catenin signalling pathway may not be associated with the inhibitory effect of cobalt on hair follicle development.

mTOR is a central regulator of cell proliferation and survival. Kellenberger and Tauchi (2013) found that mTOR phosphorylated at S2448 (p-mTOR) was localised at certain sites of hair follicle development in a phase-dependent manner. mTOR signalling is activated in hair follicle stem cells at the telogen-to-anagen transition (Deng *et al.,* 2015). Hair cycle initiation was delayed after the addition of the mTOR inhibitor rapamycin (Kellenberger and Tauchi 2013). The levels of phosphorylated mTOR and p70s6k (a downstream target of mTOR) proteins were significantly decreased by supplementation with high levels of cobalt, indicating that the mTOR/p70s6k signal may be involved in the mechanism by which cobalt regulate hair follicle development. This result is consistent with those of previous research, which suggested that cobalt causes retinal pigment epithelial cell death by suppressing mTOR activation (Li *et al.,* 2013). Inhibition of mTOR/p70S6K signalling was also associated with cobalt chloride ($CoCl_2$)-induced hypoxia damage in differentiated PC12 cells (Zhong *et al.,* 2014). Additionally, BMP signalling activity was dramatically prolonged in mTOR signalling-deficient hair follicles. mTOR signalling regulates stem cell activation during hair regeneration through neutralising BMP-mediated repression (Deng *et al.,* 2015). In our study, the opposite changes in mTOR and BMP signalling implied that mTOR signalling is involved in the regulation of BMP2/4 gene expression by high levels of cobalt.

## CONCLUSION

The present study shows that the addition of high levels of cobalt inhibits hair follicle development and that the mTOR-BMP signalling pathway may be involved in this process.

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